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Introduction

Alterations to microtubule dynamics, leading to a less stable polymer, may be an crucial determinant in the development of resistance towards Taxol, and other drugs with a binding site on the microtubule polymer. We propose that two potential mechanisms by which breast cancer cells could alter their microtubule dynamics are by (1) differential expression of the several α - and β -tubulin isotypes and (2) differential binding of endogenous regulators of microtubule assembly to the cytoskeleton as a result of posttranslational modifications to these tubulin isoforms. Currently, there is no reliable method for quantitating the complete α/β -tubulin protein isotype composition in human cells. Moreover, no precise structural information exists on the structural diversity of α - and β -tubulin isoforms in human tumors, tissue, or cell lines. The overall goal of this proposal is to develop rapid and innovative protein-based technologies for both quantitating the α - and β -tubulin isotype composition in drug-sensitive and -resistant human breast cell lines, and for characterizing the posttranslational modifications to these isoforms. Since the majority of the amino acid sequence differences, and also posttranslational modifications, occur within the C-terminal 20 amino acid residues of α/β -tubulin, we propose to develop a mass spectrometry-based methodology for the analysis of these C-terminal domains from human cell lines.

Body

Task 1 - To develop the quantitative MALDI-MS procedure to determine tubulin isotype composition.

Peptides corresponding to the C-terminal CNBr fragments of human α - and β - tubulins found in the MDA-MB-231 breast cell line, including $\kappa\alpha 1$, α^* , $\beta 1$, βII and βIVb , were synthesized on an Applied Biosystems 433A Peptide Synthesizer using Fmoc-based chemistry. All peptides were prepared on a 0.1 mmol scale. We have also prepared detyrosinated $\kappa\alpha 1$ and the phosphorylated and non-phosphorylated forms of βIII . The peptides were purified by preparative (1 x 22 cm) C8 reverse phase chromatography using acetonitrile/ 0.1% TFA gradients. MALDI-MS was used to authenticate the structure of each peptide. Figure 1 is a representative analytical C-8 reverse phase chromatogram of selected purified peptides.

We have also synthesized and purified the ^{15}N -labeled analogs of $\kappa\alpha 1$ and its detyrosinated counterpart. ^{15}N -labeled amino acids were incorporated at the underlined/bold residues, thus increasing the mass of both peptides by 4 daltons.



The MALDI-MS spectrum of the ^{14}N - and ^{15}N -labeled detyrosinated $\kappa\alpha 1$ peptide is shown in figure 2. Note the ~4 dalton difference between the largest peaks in the two ion clusters. The ^{14}N -labeled peptide has a predicted monoisotopic mass of m/z 2698.1, a difference of 0.3 m/z from the observed MALDI-TOF data. This peptide has a predicted isotope cluster as follows:

<u>m/z</u>	<u>% Relative Intensity</u>
2698.1	72.3
2699.1	100.0
2700.1	76.3
2701.2	41.8
2702.2	18.2

The observed ions starting at m/z 2701.8 corresponds to ^{15}N - labeled peptide. The observed isotopic cluster for this ^{15}N peptide agrees with the predicted isotopic cluster.

Task 2 - To optimize conditions for the CNBr release of C-terminal tubulin peptides.

In the original application we proposed radiolabeling bovine brain tubulins with ^3H and ^{32}P using tubulin tyrosine ligase and casein kinase, respectively, to optimize CNBr digestions conditions for the maximal recovery of tubulin C-terminal peptides. We have found, however, that we can avoid the need for radiolabeling of tubulin by using an antibody-based detection system. A rabbit polyclonal antibody prepared against a synthetic peptide corresponding to the final 12 amino acids of human $\alpha 1$ was used to follow the release of the CNBr C-terminal fragment. Under our published digestion conditions (3), i.e. CNBr (150 mg/ml) in 70 % formic acid for 3.5 hr at room temperature, no immunoreactivity remained on the nitrocellulose filter after the incubation period. This result was in agreement with our previous studies indicating that these digestion conditions also resulted in the quantitative cleavage of brain tubulin in solution.

Task 3 - To characterize and quantify the tubulin isotype composition in the parental drug-sensitive MDA-MB 231 and MCF-7 breast carcinoma cell lines.

Two abstracts presented at the "French-American Colloquium on the Cytoskeleton and Human Disease" in Marseille (April 2001) suggested that the β II-tubulin isotype was a major component of the tubulin cytoskeleton in the MDA-MB 231 breast cell line (1, 2). In both cases, the identification was by immunological-based methods. Our mass spectrometry-based method, which measures isotype composition based on the CNBr-release of the highly divergent C-terminal peptides, found that the β II isotype was below the level of detection in this cell line (3). Since we could detect β II tubulin in bovine brain microtubule preparations, we did not believe that our inability to detect the β II isoform in this cell line was a problem inherent to our MS-based method. Nevertheless, we decided that it was important to establish the tubulin isotype composition in MDA-MB 231 breast cell line by a process that did not involve CNBr fragmentation.

For these studies, microtubules were isolated from MDA-MB 231 cell extracts using either Taxol dependent polymerization or the glutamate-based purification of Sackett (4). Both methods yielded ~100-200 μg of tubulin from ten 100 mm culture dishes of cells. For LC/ESI-MS studies, 10 μg of tubulin sample in 70% formic acid was loaded onto a 1 mm x 15 cm Vydac C4 column (Vydac, Hesperia, CA). A HP 1100 high-performance liquid chromatography (HPLC) equipped with a degasser and a binary pump was used to degas the solvents and pump the solvents to generate acetonitrile gradients at a flow rate of 50 $\mu\text{l}/\text{min}$. Solvent A was deionized water containing 5% acetonitrile and 0.1% trifluoroacetic acid and solvent B was 95% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. The sample was desalted at 5%B for 45 min and separated by a 3 min 5%B-40%B gradient followed by a 100 min 40%B-60%B and 7 min 60%B-95%B gradients and washing at 95%B for 10 min. The column effluent was delivered directly to a LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, Riviera Beach, FL) without flow splitting. Figure 3 (top panel) is a representative chromatogram showing the total ion current of a typical human microtubule preparation. The peak centered at 52.45 min contains the α - and β -

tubulin monomers. Figure 3 (bottom panel) is the deconvoluted mass spectrum of the tubulin ion

Human Tubulin Isotype (accession #)	Predicted Mass
β I (AAD33873)	49670.82
β I (P07437)	49758.90
β II (AL050056)	49953.00
β II (0808321A)	49405.55
α -1 (AAD33871)	50135.63
α -1 (I77403)	50151.63
α -1 (NP_006000)	50157.68
α^* (mouse α -6 like)	49881.30

current shown in the top panel. Three major ions are observed with calculated masses of 49658, 49881 and 50137 daltons, respectively. The predicted masses of selected human tubulins found in the gene/protein data bases are shown in the following table. Multiple entries exist for β I, β II and α -1 tubulins. The mass of α^* -tubulin was determined by replacing *in silico* the C-terminal CNBr fragment of mouse α -6 with the corresponding peptide of this newly described α -tubulin. The ions at 49881.0 and 50137.0 are close to the predicted masses for α^* and α -1 tubulins, respectively. Furthermore, the ratio of these two alpha-tubulins (i.e., area under peaks) is close (3:7, α^* : α -1) to that observed using the CNBr cleavage method. The ion at 49658.0 approximates the mass of one of the predicted β I tubulins.

Importantly, no peaks of significant intensity are observed in the regions expected for the β II tubulins. Thus the data obtained from the analysis of native, full length tubulins are in complete agreement with the isotype composition data obtained by analysis of the C-terminal tubulin peptides released by CNBr. Both of our MS-based methods show that the β II is not a major tubulin isotype in the MDA-MB 231 breast carcinoma cell line.

Key Research Accomplishments

- Synthesis and purification of the highly divergent C-terminal peptides of the human tubulin isotypes including, $\kappa\alpha$ 1, detyrosinated $\kappa\alpha$ 1, α^* , β I, β II, β IVb, and phospho- and dephospho- β III.
- Synthesis and purification of 15 N-labeled $\kappa\alpha$ 1 and detyrosinated $\kappa\alpha$ 1.
- Demonstration that the 14 N- and 15 N-labeled peptides are clearly resolvable by MALDI-MS.
- Establish that the CNBr digestion conditions are optimal for the release of the C-terminal tubulin peptides from nitrocellulose.
- Characterization of the tubulin isotype composition of the MDA-MB 231 breast carcinoma cell line by 2 independent mass spectrometry-based methods.

Reportable Outcomes

Rao, S, Åberg, F, Deng, H., Nieves, E., Horwitz, S.B., and Orr, G.A. (2001) Analysis of tubulin isotype composition in human breast and lung carcinoma cell lines and tissue by mass spectrometry. Abstract presented at Fifth International Symposium of Mass Spectrometry in the Health and Life Sciences: Molecular and Cellular Proteomics, San Francisco, August 2001.

Verdier-Pinard, P., Martello, L., Wang, F., Orr, G.A., and Horwitz, S. B. Analysis of α - and β -tubulin content by LC-MS and IEF in human cancer cells sensitive and resistant to microtubule-stabilizing drugs. Abstract presented at the American Association for Cancer Research meeting,

San Francisco, 2002

Conclusions

Despite the clinical success of Taxol, drug resistance poses a major impediment in the treatment of malignancies with this drug. An emerging theme in studies of Taxol resistance is that alterations to microtubule dynamics may be a crucial factor in the development of resistance. Microtubule assembly can be potentially altered by differential expression and/or posttranslational modifications to tubulin isotypes. Although PCR- and microarray-based technologies can be used to measure mRNA expression levels for tubulins, there is a need for the development of rapid and sensitive protein-based methods for determining the tubulin protein isotype composition, and characterizing posttranslational modifications to these isotypes, in drug-sensitive and resistant cell lines. The data presented in this progress report represent the first analysis of native tubulins in human cell lines by mass spectrometry. Importantly, they show that determination of tubulin isotype composition by analyzing the highly divergent C-terminal peptides is a valid approach. The information gained from these experiments will provide a greater understanding of the modifications that occur to microtubules when human breast tumors become resistant to Taxol.

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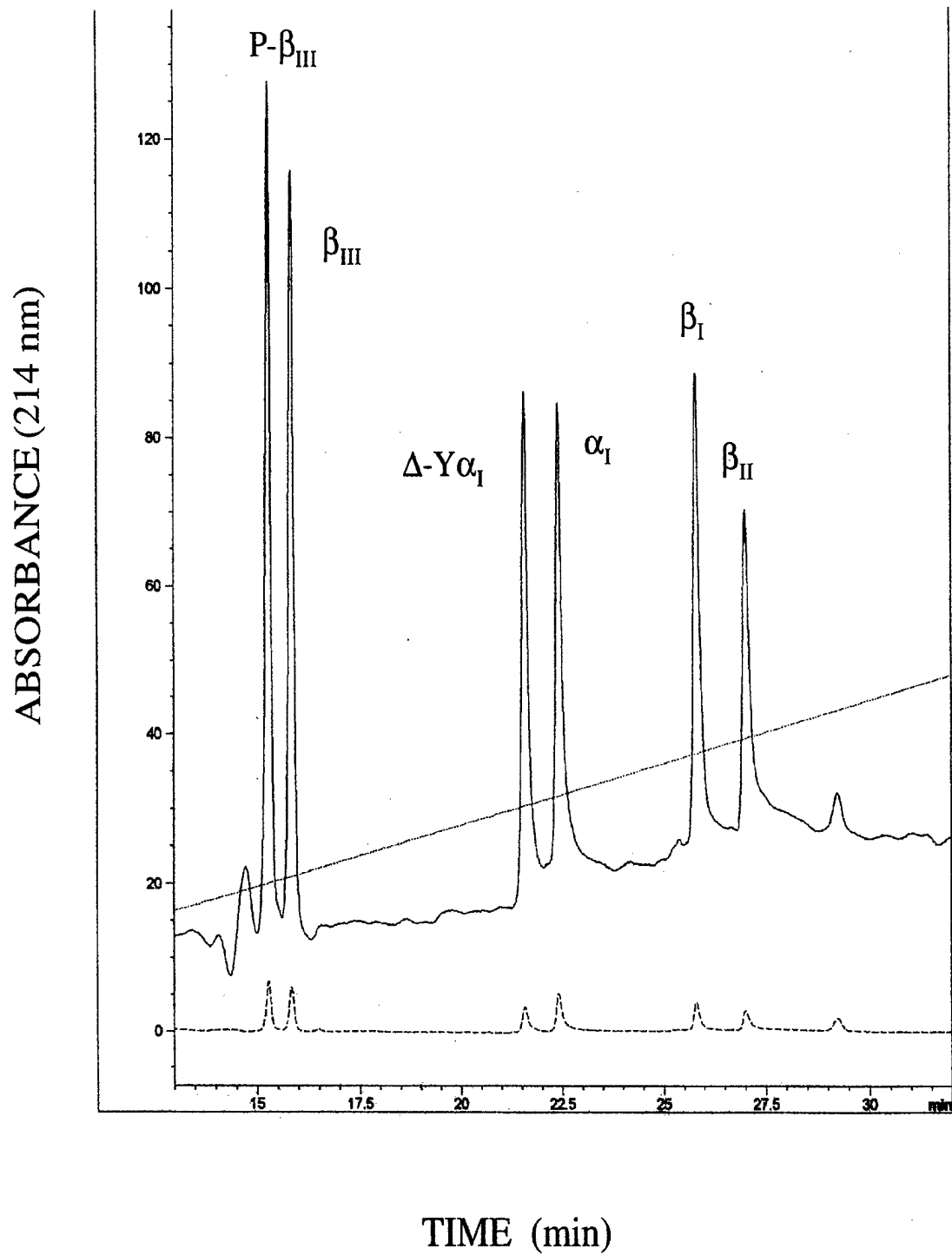


Figure 1. C-18 reverse phase HPLC of synthetic peptides corresponding to C-terminal CNBr fragments of selected human tubulins

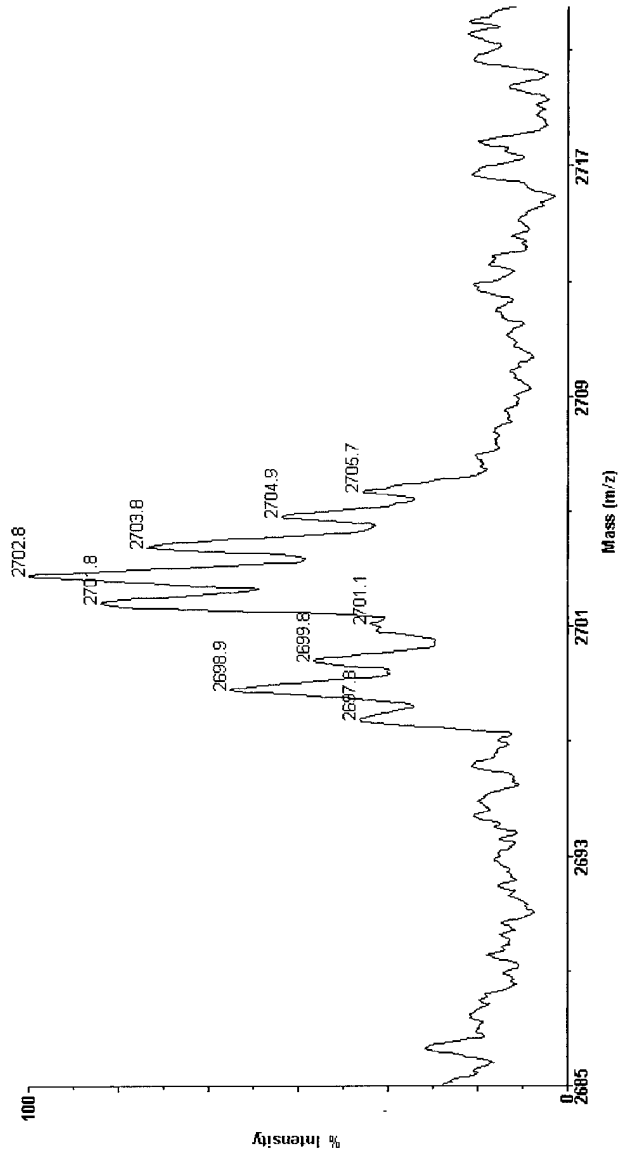


Figure 2. MALDI TOF-MS of the ^{14}N and ^{15}N -labeled synthetic detyrosinated k- α 1 peptides (AALEKDYEEVGVDSVEGEGEE).).

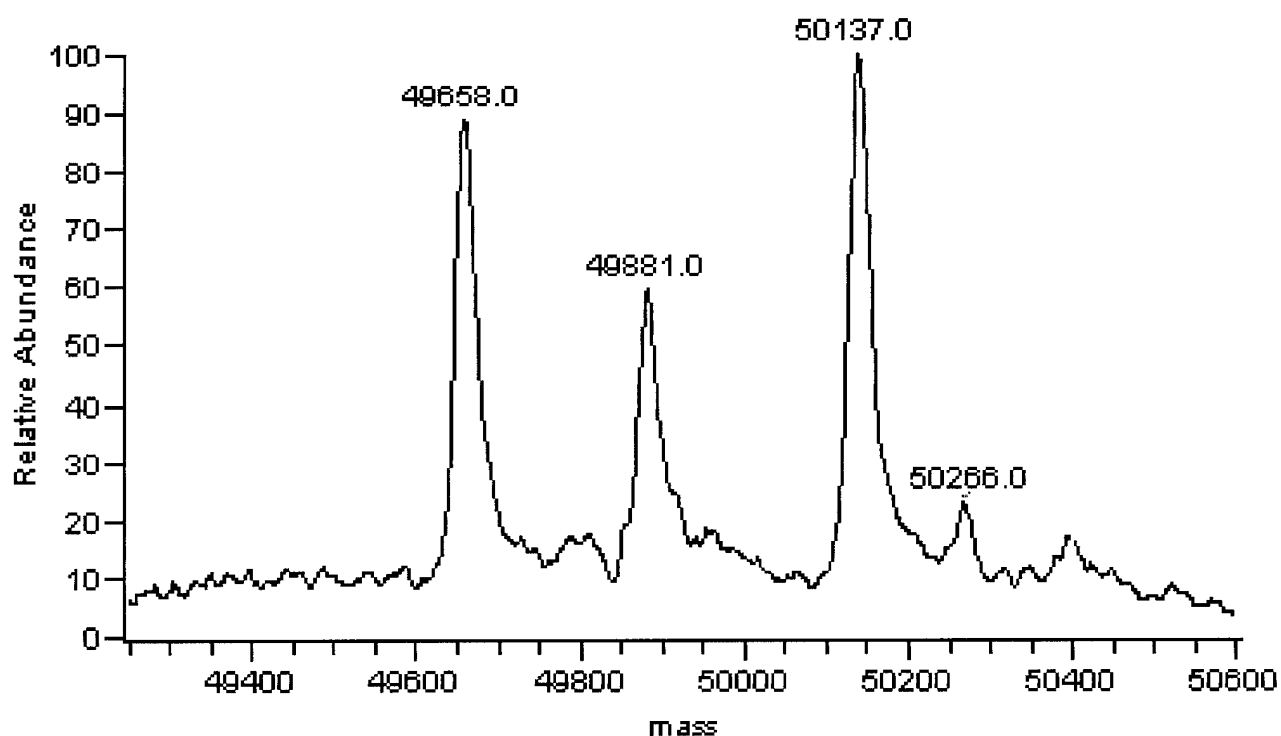
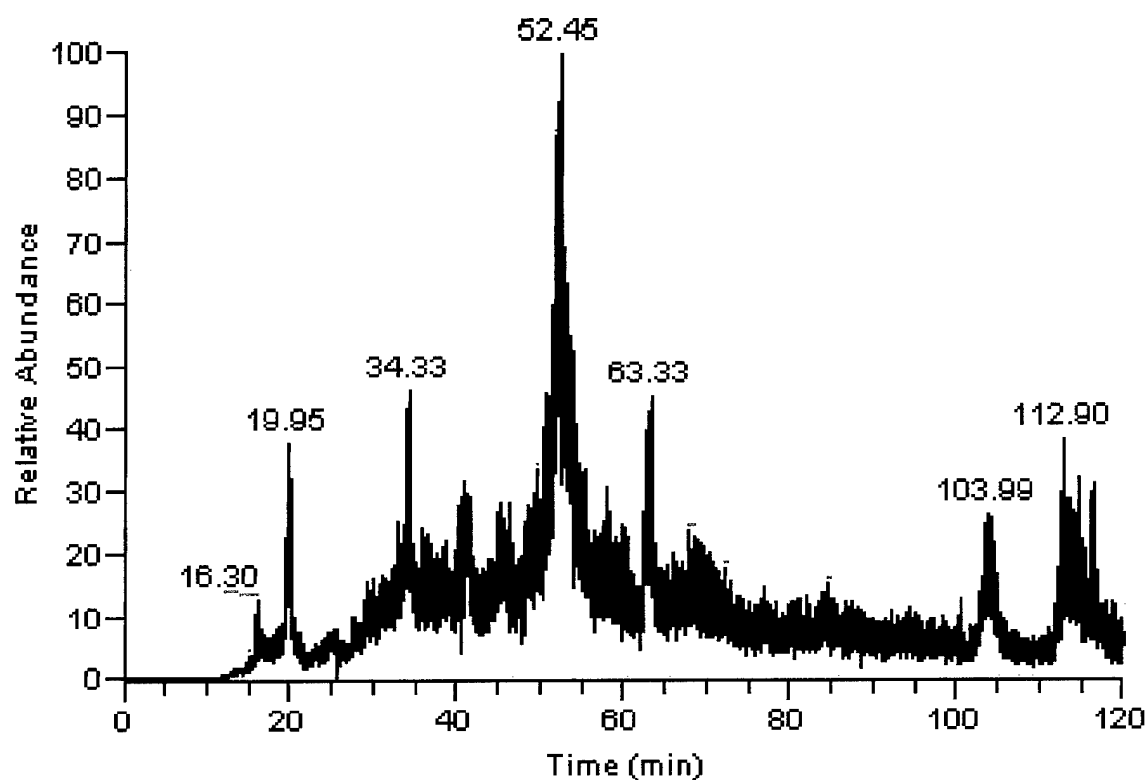


Figure 3. LC-ESI-MS analysis of human tubulins. Top panel, total ion current; Bottom panel, deconvoluted mass spectrum of ions in peak centered at 52.45 min.